Autotrophic acetyl coenzyme A synthesis in vitro from two CO_2 in *Methanobacterium*

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1. INTRODUCTION

The methanogenic bacterium *Methanobacterium thermoautotrophicum* grows on H_2 and CO_2 as sole energy and carbon sources [1,2]. In this autotroph the Calvin cycle does not operate in carbon dioxide assimilation into cell material [3]. Different experiments point instead to a novel pathway in which acetyl coenzyme A, rather than 3-phosphoglycerate, appears as the earliest detectable CO_2 fixation product [4]. This acetyl CoA is not derived from pyruvate, but rather is synthesized via unknown one-carbon intermediates, which are derived from two different CO_2 fixations. We have shown that growing cells of *M. thermoautotrophicum* incorporate carbon monoxide into the carboxyl group of acetyl CoA [5], indicating that total synthesis of acetyl CoA in methanogenic bacteria might be mechanistically related to acetate formation in acetogenic bacteria [6,7].

This paper presents an in vitro system of autotrophic [^{14}C]acetyl CoA formation from two $^{14}CO_2$ in *M. thermoautotrophicum*, and shows that acetyl CoA rather than acetate is the initial fixation product. Both acetyl CoA synthesis and methane formation were greatly stimulated by the addition of methyl coenzyme M. The acetate moiety formed from $^{14}CO_2$ was equally labeled in the C-1 and C-2 positions. Acetyl CoA synthesis was inhibited by low concentrations of cyanide, which had no significant effect on methane formation. This inhibition was reversed by carbon monoxide.

This is the first demonstration of acetyl CoA formation in vitro from 2 CO_2 in methanogenic bacteria. Furthermore, the data are consistent with the hypothesis that cyanide-sensitive carbon monoxide dehydrogenase is involved in acetyl CoA synthesis.

2. MATERIALS AND METHODS

Methanobacterium thermoautotrophicum (Marburg strain) was grown as in [8]. Cells were harvested at $\Delta A_{578} = 3.5$ and stored at $-60°C$ until use.

2.1. *In vitro system of acetyl CoA formation*

The assay was performed under anaerobic conditions in a stoppered glass vial with 4.5 ml gas phase and 0.4 ml assay mixture (pH 6.7, 60°C) containing 130 mM PIPES, 10.5 mM $MgCl_2$, 2.6 mM ATP, 13 mM CH_3SCoM, 5 mM DTT, 0.26 mM CoA, cell-free extract (10 mg protein) or 0.2% (w/v) Triton X-100 plus a suspension made up of frozen cells (10 mg protein). The gas phase was 80% H_2/20% $^{14}CO_2$ (1800 dpm/nmol total CO_2) at 0.2 bar overpressure. After a 10 min preincubation period at room temperature the assay was incubated for 30 min in a shaking water bath at 60°C and then stopped by addition of 0.3 ml 2 M $HClO_4$. Methane in the gas phase was quantitated gas chromatographically [9].

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2.2. *Isolation and degradation of acetate from acetyl CoA*

[^{14}C]Acetyl CoA formed in the assay was hydrolyzed by 30 min incubation at pH > 12, 50°C. [^{14}C]Acetate supplemented with 10 μmol carrier acetate was purified by 40 min extraction with diethylether in a Kutscher-Steudel apparatus, followed by ion-exchange chromatography on 1.5 g Dowex 1 × 8, 100–200 mesh, formate form. The column was washed subsequently with 2 ml water, 11 ml 0.05 M formic acid, 15 ml 0.4 M formic acid and 15 ml 1 M hydrochloric acid. [^{14}C]Acetic acid was eluted by 0.05 M formic acid (fig.1). The [^{14}C]acetic acid fractions were pooled, formic acid was oxidized by Hg^{2+}-treatment, and [^{14}C]acetic acid was reisolated by steam distillation. [^{14}C]Acetate recovery was ~70%, as determined by use of authentic [^{14}C]acetyl CoA and by enzymatic measurement of the carrier acetate. The [^{14}C]acetate was chemically degraded according to the Schmidt procedure [11]. [^{14}C]Acetyl CoA formed in the assay was also trapped as [^{14}C]citrate with citrate synthase according to [12]. The [^{14}C]citrate was isolated by 4 h ether perforation at pH 2, followed by ion-exchange chromatography (see fig.1). [^{14}C]Acetate was released by citrate lyase [12] and purified as above. Acetate was determined enzymatically with acetate thiokinase. Radioactivity determination was by liquid scintillation counting.

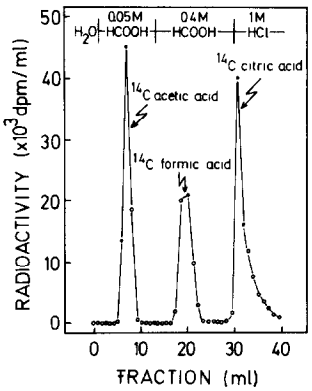

Fig.1. Elution of authentic [^{14}C]acetic acid (78000 dpm/10 μmol) [^{14}C]formic acid (55000 dpm/10 μmol) and [^{14}C]citric acid (94000 dpm/10 μmol) in 1 ml fractions from 1.5 g Dowex 1 × 8, 100–200 mesh, formate form. Values in brackets indicate radioactivity and quantity of each compound applied to the column.

3. RESULTS

Triton X-100 incubated cells of *M. thermoautotrophicum* synthesized both [^{14}C]methane and [^{14}C]acetyl CoA from $^{14}CO_2$ at 60°C under strictly anaerobic conditions. During 30 min incubation at 60°C 14 μmol [^{14}C]methane and 20 nmol [^{14}C]acetyl CoA were synthesized (table 1). During 10 min preincubation at room temperature <3% of these amounts were formed. Cell-free extracts gave comparable results (not shown). Methane and acetyl CoA formation were linearly dependent on incubation time (0–30 min) and amount of protein added (0–10 mg). The [^{14}C]acetate moiety of [^{14}C]acetyl CoA was routinely isolated from perchloric acid stopped assay solution by alkaline hydrolysis, followed by ether perforation as in section 2. The ether-perforable material contained about 85% [^{14}C]formate and 10% [^{14}C]acetate, which was clearly separated from all other radioactive compounds (e.g., [^{14}C]formate) by ion-exchange chromatography. A standard elution profile is depicted in fig.1. [^{14}C]Acetyl CoA was identified as a reaction product by trapping it into [^{14}C]citrate. [^{14}C]Citrate cannot be formed from $^{14}CO_2$ in *Methanobacterium* [4]. After isolation and cleavage of [^{14}C]citrate by citrate lyase,

Table 1

Synthesis of [^{14}C]acetyl CoA and [^{14}C]methane from $^{14}CO_2$ by Triton X-100-treated cells of *M. thermoautotrophicum*

Assay conditions	[^{14}C]Acetyl CoA formed (nmol)	[^{14}C]Methane formed (μmol)
− CH_3SCoM	<0.5	<0.2
+ CH_3SCoM	20	14
+ CH_3SCoM + KCN	0.5	13
+ CH_3SCoM + KCN + CO	14	13

Effects of methyl CoM (13 mM), cyanide (0.2 mM) and carbon monoxide (10% gas phase) are shown. [^{14}C]Acetyl CoA formation was calculated by using the specific radioactivity of $^{14}CO_2$ (1800 dpm/nmol). Further assay conditions are given in section 2

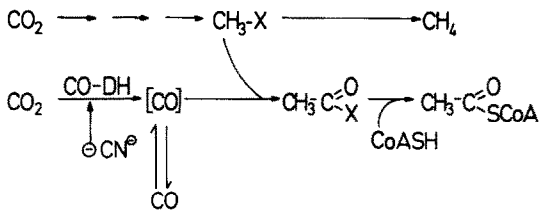

Fig.2. Tentative scheme of autotrophic acetyl CoA synthesis from CO_2 in *Methanobacterium*. Cyanide sensitive carbon monoxide dehydrogenase [CO-DH] and an intermediate deriving from CO_2 reduction to methane may be involved as indicated.

[^{14}C]acetate was reisolated. This test for [^{14}C]acetyl CoA formation was specific, since [^{14}C]acetate could not be reisolated from parallel samples in which citrate synthase was omitted. By this procedure ~14 nmol [^{14}C]acetyl CoA were recovered as [^{14}C]acetate, as compared to 20 nmol isolated by the standard procedure. When label distribution in the [^{14}C]acetate molecule was investigated by Schmidt degradation, it was found that each carbon atom contained 50% of the total label fixed from $^{14}CO_2$ (not shown). The standard assay contained ATP, Mg^{2+}, coenzyme A and CH_3SCoM. Omission of CH_3SCoM resulted in a marked decrese in [^{14}C]methane and [^{14}C]acetyl CoA formation (see table 1). Less than 5% of each compound was synthesized as compared with standard conditions. Additions of 0.2 mM potassium cyanide to the complete assay during the preincubation resulted in >90% inhibition of [^{14}C]acetyl CoA formation, whereas methane formation was not affected. This inhibition of acetyl CoA synthesis was reversed by addition of 10% carbon monoxide to the H_2/CO_2 gas phase (table 1).

4. DISCUSSION

In *M. thermoautotrophicum*, acetyl CoA synthesis occurs via condensation of 2 one-carbon units which derive from 2 different CO_2 fixations. The identification of [^{14}C]acetyl CoA, as opposed to free acetate, as the product of the reaction was accomplished by specifically trapping acetyl CoA into citrate. Citrate is lacking in cell carbon metabolism of *M. thermoautotrophicum* [4]. Although the rates of in vitro acetyl CoA synthesis and methane formation were low as compared to in vivo rates, our values for methane formation in vitro are similar to those in [13]. The factors that cause this discrepancy between in vivo and in vitro rates are not yet known. Methane production and acetyl CoA synthesis were both stimulated by addition of methyl CoM. This result suggests that these processes might be coupled or possess one or more common intermediates. One candidate for a common intermediate is CH_3SCoM, which might be identical to CH_3-X in fig.2. In [14,15] a similar mechanism was discussed for acetate conversion to CH_4 in *M. barkeri*. In this case, one would expect a dilution of radioactivity in the methyl group of acetate by carbon assimilation from unlabeled CH_3SCoM, since the carboxyl group of the molecule derives exclusively from $^{14}CO_2$. However, our data exclude that a substantial amount of CH_3SCoM was converted into C-2 of acetate.

There is evidence that the carboxyl group of acetate derives from CO_2 via reduction to a bound carbonyl which exchanges with gaseous CO [5]. We proposed that this reduction is catalysed by CO dehydrogenase [5], which is active in *M. thermoautotrophicum* even when this organism is grown in the absence of CO. In [9] this enzyme was inactivated by low concentrations of cyanide. These findings are consistent with our observations that cyanide inhibits in vitro acetyl CoA synthesis and that CO reverses this inhibition. These experiments lead us to propose that CO dehydrogenase functions in *M. thermoautotrophicum* CO_2 assimilation (fig.2).

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